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14. ABSTRACT

The purpose of this study was to determine if CHFR was biologically relevant to breast cancer characteristics and progression. Here, we studied both breast cancer cell lines and primary samples from breast cancer patients to investigate CHFR as a relevant tumor suppressor in breast cancer and to associate CHFR expression with clinical and pathological variables. A large percentage of samples demonstrated negative or weak CHFR protein expression or staining. In addition, lack of CHFR detection correlated with increased tumor size in patients and was weakly associated with ER-negative primary tumors. To study the effects of low CHFR expression in vitro, we decreased CHFR gene expression in mammary epithelial cells. Notably, this resulted in the acquisition of many phenotypes associated with malignant progression including higher growth rates, increased mitotic index, enhanced cellular invasion and motility, morphological changes, and aneuploidy. Considering the association between CHFR expression and breast cancer tumor size, the in vitro data presented here, and its previously published correlation with cellular response to chemotherapeutics such as Taxol, this study provides substantial evidence to identify CHFR as an important tumor suppressor in breast cancer and possibly a biomarker for tumor response to microtubule-targeting drugs like Taxol.

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cell cycle, mitotic checkpoints, CHFR, chemotherapeutic response, and tumorigenesis

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Introduction:

CHFR (Checkpoint with FHA and Ring Finger) is hypothesized to mediate a delay in cell cycle progression early in mitosis in response to microtubule stress. One example of microtubule stress results from exposure to the chemotherapeutic drug Taxol, which is often used to treat breast cancer patients. As a potential regulator of cell cycle progression and drug response, CHFR naturally becomes an interesting target for study in breast cancer. Little is known about the molecular functions and signaling pathways that CHFR mediates, but it has been reported to frequently show decreased or lost gene expression in cancer cells when compared to normal cells from the same tissue. The purpose of this study was to characterize the role of CHFR in breast cancer tumorigenesis using both cultured breast cancer cell lines and primary breast cancers from patients. In particular, the research presented here analyzed the expression of CHFR protein in cell lines and primary tumors and then tried to correlate protein expression with clinical and pathological information from primary samples. In addition, cell culture models were used to determine if changing *CHFR* expression would result in the cells becoming more like cancer cells instead of normal mammary epithelia.

Report:

Task 1: Determine if CHFR protein expression is decreased or lost in breast cancers and if its expression has prognostic value using both mammary epithelial cell lines and primary matched normal and tumor tissues. (Months 1-15)

Outstanding progress has been made on Task 1. Initial analysis of breast cancer cell lines and primary breast cancer specimens for CHFR expression has largely been completed. Results from these important studies suggest that the loss of CHFR expression is associated with other clinical variables as described below.

Task 1a: Use Western blot analysis with a newly developed polyclonal antibody to analyze CHFR protein expression in approximately 30 immortalized and transformed mammary epithelial cell lines (months 1-4).

Western blotting was performed using whole cell lysates from a panel of unsynchronized breast cancer cells and immortalized ("normal") human mammary epithelial cells (HMECs). A custom-made polyclonal antibody against the N-terminus of the CHFR protein was developed and used to detect CHFR expression in the cell lines and a beta-Actin antibody was used as a control to show that equal amounts of protein were used for each sample. Preliminary evidence indicated that 37.5% of breast cancer cell lines had CHFR expression lower than that observed in the HMEC lines (Fig. 1A, *appendix*). We have since determined that this antibody was not as robust for other applications, such as immunohistochemistry. Thus, a new anti-CHFR monoclonal antibody from Abnova Corporation will be used in repeat immunoblot experiments so that the Western blot data can be more readily compared to immunohistochemistry data of primary tumors. Fresh cell lysates are being prepared from our large panel of breast cancer cell lines to repeat the Western Blot analysis. I estimate this additional task will be completed in three weeks .

Task 1b: Perform immunohistochemistry on matched normal and breast cancer tissues obtained from patient samples from the University of Michigan (months 4-12).

The second portion of this task was to assess CHFR protein in primary breast cancers compared to normal tissues. Assessment of CHFR expression in matched primary normal and tumor tissues to determine if expression is altered between the two states is underway. This portion of task one should be completed within in the next two months.

Task 1c and 1d: Conduct tissue microarrays to detect CHFR expression across a range of tumor grades and stages (months 8-14). Perform statistical analysis (Fisher's exact test and Chi square tests) to determine if the loss of CHFR expression is correlated with different stages of tumorigenesis (months 14-15).

I have used immunohistochemistry with the monoclonal CHFR antibody from Abnova to detect CHFR in 160 primary invasive breast cancer samples from patients at the University of Michigan utilizing a breast cancer tissue microarray. Initially, we found that 36% of primary invasive breast cancers had completely lost CHFR expression (similar to the results seen in our panel of breast cancer cell lines), compared to only 5% of samples that showed strong expression. Using the statistical analysis of the Wilcoxon Rank Test for significance and Fisher's Exact test, we looked for correlations between CHFR staining by immunohistochemistry and clinico-pathological variables of the tumors. It was determined that positive CHFR protein expression was very strongly associated with small tumor size, a good prognostic indicator of early tumor stage, and it was weakly correlated with an estrogen receptor-positive status, which indicates a course of action for treatment (Fig. 2B, appendix). This is an important finding that suggests CHFR expression may be a useful biomarker for malignant progression and therapeutic response. Please see the manuscript draft included in the appendix for details on methods and results.

<u>Task</u> 2: Examine if CHFR expression correlates with cancer phenotypes in vitro (Months 12-25)

We are excited about the significant progress I have made on this task as described below. I have accelerated efforts on this task to move ahead of schedule given the high clinical relevance CHFR expression may have to breast cancer as found through the above experiments.

Task 2a: Design a retroviral vector that will have controlled, inducible expression of CHFR, infect breast cancer cell lines (Hs578t and BT20) with the retrovirus carrying the construct, and select for stable transformants (months 12-17)

Gene cloning is currently underway to create a system in which CHFR expression can be controlled, turned on or off within the cell, using a tetracycline responsive promoter. This will allow us to better correlate the amount of CHFR with cellular phenotypes. In addition, creating this gene construct will make it easier to determine what happens to a breast cancer cell when CHFR expression is re-introduced, since it has proven extremely difficult to overexpress CHFR in cancer cells; high expression appears to be toxic. However, preliminary evidence suggested that overexpressing CHFR in a breast cancer cell line, Hs578t, can reverse some of the tumorigenic phenotypes of that cell, particular invasive potential and motility (Fig. 5, appendix). This was the only breast cancer cell line to date that is capable of overexpression after transfection of a full-length cDNA encoding CHFR. We think more significant results will be evident if the amount of CHFR that is overexpressed can be titrated so as to prevent cellular toxicity. The creation of the tetracycline-regulated construct for CHFR, its introduction into breast cancer cell lines, and the subsequent phenotypic analyses will require approximately four to six months, at least, to accomplish.

Task 2b: Design a retroviral vector that will express a siRNA construct targeted against CHFR to decrease CHFR expression in "normal" immortalized mammary epithelial cell lines (MCF10A and HPV 1-30). Transduce cell lines with the retrovirus construct and select for stable clones (months 12-17).

To accomplish this task, a stable short hairpin RNA (shRNA) construct with partial complementarity to CHFR was introduced into two HMEC cell lines, MCF10A and HPV4-12, using retroviral transduction to permanently decrease CHFR expression by approximately 80%. In addition, a second approach was used to confirm the results observed in the above cell lines using a small, interfering RNA (siRNA) construct against CHFR to temporarily decrease expression by about 95%; this would allow us to determine the immediate affects of CHFR loss while circumventing the complications that

may arise when cells are permanently changed in culture over extended periods of time (Fig. 3A, appendix).

Task 2c: Analyze transduced cell lines (see above) for changes in cancer-related phenotypes: (1) foci formation, (2) Matrigel invasion assay, (3) migration assay, (4) changes in growth rate, (5) changes in mitotic index (ratio of cells undergoing mitosis at a given timepoint), (6) alterations in genomic stability/ploidy status, and (7) response to treatment with nocodazole, and paclitaxel (months 17-25)

We tested the altered cell lines, with drastically less CHFR as described above, to determine if any of their characteristics had changed to look more like breast cancer cells instead of normal epithelial cells. We tested both of the cell lines, HPV4-12 and MCF10A, using several cell culture-based assay.

By counting the numbers of cells over the course of about a week, it was determined that cells in which CHFR expression was stably decreased by shRNA grew much faster than their untransduced, parental counterparts and the negative controls, which had a non-specific shRNA construct (Fig. 3B, appendix). The cells without CHFR, whether it was a permanent or a temporary loss, also became more invasive, using a Matrigel invasion assay (Fig. 4A, appendix). Cells without CHFR also became more motile when compared to controls (Fig. 4B, appendix). Intriguingly, both cell lines, HPV4-12 and MCF10A, acquired increased numbers of misshapen nucleoli when CHFR was absent; this phenotype strongly correlates with a poor patient prognosis when it is observed in primary cancers (Fig. 4C. appendix). In addition, only the MCF10A cell line underwent a morphology change so that it appeared mesenchymal instead of epithelial when CHFR was absent (Fig. 4D, appendix). This is a cellular transition that is sometimes observed in primary breast cancers. Finally, cytogenetic analysis revealed that both HPV4-12 and MCF10A cells became aneuploid without CHFR, indicating that CHFR is important in maintaining genomic stability in normal cells (Fig. 6A and 6B, appendix). Together, these results suggest a critically important role for CHFR expression in mammary tumorigenesis. Details on the methods and results from these experiments are included in the text of the manuscript included in the appendix.

In the next year, the phenotype of genomic instability will be analyzed more closely to determine how cells without CHFR become incapable of properly separating their chromosomes during mitosis. There are many ways in which normal cells can lose their ability to maintain proper chromosome number, so it important to the cellular and molecular characterization of CHFR and these cell lines to determine how this phenotype happens.

In addition, within the next six months we will continue to analyze the phenotypic changes that occur in these two HMEC lines when CHFR expression is dramatically decreased. Preliminary data indicates that when CHFR expression is lowered, the cells show an increase in the number of mitotic cells in population, which is measured as the number of cells that stain positive for phosphorylated Histone H3 on residue serine28 (Fig. 3C, appendix). This phosphorylation event occurs immediately after the CHFR-mediated checkpoint, so it is not only an indication of mitotic index, but it also permits an analysis of whether or not the checkpoint is intact[1]. This experiment requires several more repetitions than what is presented in Figure 3C in order to test for statistical significance. Another phenotype to be assessed in HMECs with lowered CHFR expression is their response to microtubule poisons, such as nocodazole and the chemotherapeutic drug paclitaxel (Taxol). The reagents and protocols for this experiment are currently being optimized. We intend on using annexin V staining of the cell membrane as an indicator of apoptosis to determine if cells without CHFR undergo more or less cell death in response to drug treatment[2]. Finally, at least one month will be dedicated to determining if HMECs with reduced CHFR expression can form foci, or mounds of cells (like a tumor), after prolonged growth in culture.

<u>Task 3</u>: Characterize CHFR as a cell cycle checkpoint protein in mammary epithelial cells (Months 24-36)

Task 3a: Perform Western blot and immuno-fluorescence to determine expression and cellular localization of CHFR at different stages of the cell cycle in mammary epithelial cell lines (months 24-26) .

The experiments for this task are still in the planning stages. I am currently cloning a CHFR full length cDNA into an inducible expression vector to create a fusion protein with GFP . This will allow easy detection of CHFR localization at different stages of the cell cycle, in addition to using live-cell imaging to determine if CHFR changes localization in response to cell cycle stage or drug treatment. In addition, cell cycle synchronization methods are still being researched to determine how to best analyze CHFR expression levels across the cell cycle. I am working closely with out Live Cell Imaging Core in developing reliable and robust experiments.

Task 3b: Find novel interacting partners with CHFR using GST pull-down and confirm with immunoprecipitation experiments (months 26-36).

Initial attempts to complete this task have been attempted. To date, the results are inconclusive as there have been a few technical difficulties. Specifically, I am still optimizing conditions for these protocols using the CHFR antibody and GST fusion protein to find the proteins that interact with CHFR in a specific and sensitive manner. Experiments for this work will probably require an additional year before the task is completed and will involve finding the proper hybridization conditions, antibodies, and stringencies for the washing buffers in the protocols.

Task 3c: Test cellular responses of cells with and without CHFR to other chemotherapeutic drugs (months 30-36).

The cells to be used for this task were generated for task 2, described above. The experiments for this task are still being planned and will be addressed in the future.

Key Research Accomplishments:

Work on this project over the past year has lead to noteworthy novel results that highlight the relevance of CHFR expression to breast cancer. Specifically, I have:

- Determined that positive CHFR expression correlated with small tumor size in primary patient samples of breast cancer. Small tumor size is one of the indicators of early tumor stage.

- Found that decreasing CHFR expression in two different mammary epithelial cell lines ("normal" cells) resulted in the cellular changes reminiscent of a cell becoming like a cancer cell. Decreased/lost CHFR expression led to:
    - Increased growth rates
    - Increased mitotic index (ie: more cells dividing in the population)
    - Morphology changes from epithelial to mesenchymal
    - Increased invasive potential
    - Increased cellular motility
    - Aneuploidy (extra chromosomes)

Reportable Outcomes:

***Manuscript*:**

Privette, L.M, Gonzalez, M.E., Ding, L., Kleer, C.G., and Petty, E.M., "Altered expression of the early mitotic checkpoint gene, *CHFR*, in breast cancers: Implications for tumor suppression," *in revision, Cancer Research*

***Presentations*:**

National Meetings:

Poster: "The Cell Cycle" National Meeting at Cold Spring Harbor, NY. May 2006

Local Meetings:

Posters: UM Cancer Center Fall Research Symposium, November 2006
Genetics Training Grant Annual Symposium, June 1, 2006
UM Dept. of Human Genetics, 50th Anniversary Symposium, May 26, 2006
UM Department of Internal Medicine Annual Symposium May 20, 2006

Seminars: Human Genetics Student Research Colloquium, April and December, 2006
Human Genetics Annual Department Retreat, September 2006

Conclusion:

Great progress has been made towards completing the first two tasks listed in the statement of work. In fact, I have accelerated progress on Task 2. Of importance, it has been discovered that CHFR expression correlates with some clinico-pathological variables important to breast cancer. In particular, CHFR expression had a strong, positive correlation was small tumor size, which is a contributing factor in determining early tumor stage, and therefore indicates that positive CHFR expression may be associated with a favorable prognosis. In addition, there was a weak correlation between positive CHFR status and estrogen receptor-positive (ER+) tumor status. This is particularly interesting because previous studies have shown that, like CHFR positive cells, ER+ cancer cells do not respond to Taxol treatment, which is why anti-estrogen therapy is so effective. This indicates that there may be a common biological system between CHFR function and estrogen receptor function or signaling. Both of these correlations with CHFR provide great evidence that CHFR is biologically relevant to breast cancer pathogenesis.

Even stronger proof that CHFR is important in breast cancer comes from the studies in task two from the statement of work, which described the phenotypic changes that a fairly normal cells undergoes when it experiences an extreme decrease (near loss) of CHFR expression. Two different HMEC lines, using two different methods to decrease expression, revealed that cells with a significantly lowered amount of CHFR began to grow faster, became more invasive and motile, underwent changes in nucleoli numbers and cellular morphology, and became aneuploid. All of these changes mimic those that a normal cell undergoes in the patient to develop into a tumor. It was incredibly interesting to realize that CHFR loss had such a great impact on invasive potential and motility *in vitro;* this indicated that CHFR may be important in regulating tumor metastasis in the patient, a finding that had never before been associated with CHFR.

The data presented here shows that 37% of cell lines have lost protein expression and that 36% of primary invasive cancers are negative for CHFR; this agrees with the preliminary findings presented in the original proposal in which many breast cancer cell lines showed low or lost *CHFR* mRNA expression. In addition, preliminary data from the original proposal indicated that breast cancer cell lines with low CHFR expression tended to have a high mitotic index. This is supported by our preliminary evidence that showed HMECs with lowered CHFR expression had a higher percentage of cells that bypassed the CHFR checkpoint and entered mitosis, as evidence by phosphor-H3-Ser28 staining by immunofluorescence. The combination of initial evidence presented in the proposal with the new findings described here indicate that CHFR contributes significantly to maintaining genomic stability and normal cellular function and, more importantly, appears to have a great tumor suppressor function in mammary tissues.

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2. Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V, *J Immunol. Methods*, 1995, 184(1):39-51

<u>Appendices</u>: (Manuscript in revision for *Cancer Research* journal)

Altered Expression of the Early Mitotic Checkpoint Gene, *CHFR*, in Breast Cancers: Implications for Tumor Suppression

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Abstract

CHFR (Checkpoint with FHA and Ring Finger) is hypothesized to mediate a delay in cell cycle progression early in mitosis in response to microtubule stress, independently of the spindle assembly checkpoint. As a potential regulator of cell cycle progression, CHFR naturally becomes an interesting target for studying cancer cells. In recent years, there has been increasing evidence supporting the role of CHFR as a tumor suppressor, most of which report loss of expression, occasionally due to promoter hypermethylation, in cancers compared to patient-matched normal tissues. Here, we studied both a panel of breast cancer cell lines as well as primary tissue samples from breast cancer patients to investigate CHFR as a relevant tumor suppressor in breast cancer and to determine whether CHFR expression was associated with clinical and pathological variables. We report that 37.5% of cell lines and 36% of patient samples demonstrated low or negative CHFR protein expression or staining. In addition, lack of CHFR detection was associated with increased tumor size and estrogen receptor (ER)-negative tumors from patients. To study the effects of low CHFR expression *in vitro*, we stably expressed a shRNA construct targeting CHFR in two lines of immortalized human mammary epithelial cells (IHMECs). Notably, decreased CHFR expression resulted in the acquisition of many phenotypes associated with malignant progression including higher growth rates, increased phosphorylation of histone H3-Ser28 (as an indicator of mitotic index), enhanced invasiveness, increased motility, and aneuploidy, further supporting the role of CHFR as a tumor suppressor in breast cancer.

Introduction

Breast cancer, the second leading cause of cancer-related death among women in the United States, is often associated with defects in cell cycle checkpoint regulation. CHFR (Checkpoint with FHA and Ring Finger) is a checkpoint protein that reportedly initiates a cell cycle delay in response to microtubule stress during prophase in mitosis (1). This delay is thought to occur prior to chromosome condensation by excluding cyclin B1 from the nucleus (2). One form of microtubule stress is treatment with taxanes such as nocodazole or paclitaxel (Taxol), a chemotherapeutic drug used for cancer patients including those with breast cancer (3). Therefore, CHFR has been hypothesized to be a tumor suppressor with a potential role as a biomarker for chemotherapeutic response to Taxol (4, 5). Many reports have noted that cancer cells that have lost *CHFR* expression are more likely to undergo apoptosis in response to microtubule poisons, which strongly supports this hypothesis (1, 5-7). The molecular mechanism by which CHFR initiates a cell cycle arrest is debated, though evidence implicates the p38/MAPK pathway, an Aurora A interaction, and/or through regulation of PLK-1 (8-11).

There is evidence that *CHFR* may function, in part, as a tumor suppressor gene. Most notably, several groups have shown that *CHFR* mRNA expression is lost or decreased in primary tumors and cancer cell lines when compared to matched normal tissues and cells. The best characterized means of expression loss is promoter hypermethylation, which occurs in a subset of tumors and cell lines and the frequency of which appears to be dependent on the tissue of origin (4, 5, 12-20). Further support that *CHFR* may mediate tumorigenesis is that its chromosomal location, 12q24, is a site for allelic imbalance and chromosome rearrangements in several types of cancer (21-25). In addition, Yu *et al.* recently published their description of a *Chfr* knockout mouse (11). The null mice were prone to developing tumors and mouse embryonic fibroblasts were aneuploid, suggesting a role for CHFR in genomic stability. However, to date there has been little functional evidence describing *CHFR* as a tumor suppressor in a human model system.

To characterize the role of CHFR in breast cancer, we used both cultured breast cell lines and primary patient samples. We assessed the expression of CHFR protein and mRNA in a panel of breast cancer cell (BCC) lines and found that expression is low or absent in many of them when compared to immortalized human mammary epithelial cells (IHMECs). Analysis of a tissue microarray composed of primary invasive breast cancer samples indicated that a significant number of patient samples showed negative or weak CHFR protein staining by immunohistochemistry and that CHFR staining was inversely correlated with tumor size. In view of this evidence that CHFR may be a tumor suppressor, we mimicked cellular loss of expression via stable shRNA and transient siRNA targeting CHFR in two IHMEC lines. This decrease in expression led to the acquisition of many phenotypes associated with malignant progression.

Methods

Cell Culture

Most cell lines were obtained from the American Type Culture Collection (AATC, Manassas, VA) and grown under recommended conditions. SUM1315-MO2, SUM102-PT, SUM190-PT, SUM159-PT, SUM149-PT, SUM52-PE, SUM185-PE, SUM225-CWN, SUM229-PE, and the human papilloma virus (HPV)-immortalized series of non-tumorigenic mammary cell lines were developed and provided by S.P. Ethier (now available from Asterand, Detroit, MI) and cultured according to specified conditions (26).

For retroviral transduction, PT67 packaging cells were transfected using FuGENE 6 with 10.0 ug of pRNA-H1.1/Hygro vector (GenScript Corp, Piscataway, NJ) containing either a scrambled sequence or a CHFR shRNA construct targeting nucleotides 324-344, 1491-1511, or 2497-2517 (accession no. AF170724). We used the pLPCX retroviral vector for overexpression of full-length *CHFR* in Hs578t cells (Clontech Laboratories, Mountainview, CA). Virus was collected after 48 hours and purified with a 0.45 micron filter. Equal parts of retrovirus-containing media and normal growth media were added to $1x10^6$ cells. Fresh media was added 24 hours later and selection with 20.0 ug/ml hygromycin (pRNAH1.1) or 1.5 ug/ml puromycin (pLPCX) began 48 hours post-infection. The resulting polyclonal

cell population stably expressing the CHFR construct(s) was subsequently used for experimentation. MCF10A cells were transduced with all three shRNA constructs whereas HPV4-12 cells were transduced with the shRNA construct targeting nucleotides 324-344 to achieve maximum knockdown.

Transient transfection of siControl or a pool of four siRNAs targeting CHFR (si*GENOME*, Dharmacon RNA Technologies, Lafayette, CO) was performed according to manufacturer's instructions. HPV4-12 cells were transfected using Dharmafect2 lipofection reagent and MCF10-A cells with Dharmafect1. For both methods, stable shRNA and transient siRNA, knockdown of CHFR expression was confirmed using semi-quantitative duplex RT-PCR and Western blotting, followed by densitometry.

Western Blotting

To assess CHFR protein levels in asynchronous cells, 50.0ug of total protein from 70-80% confluent cell cultures was separated on 7.5% Tris-HCl mini-gels (Bio-Rad Laboratories, Hercules, CA) and immunoblotted to Hybond-P PVDF membrane (Amersham Biosciences, Piscataway, NJ). Blots were blocked for one hour in 5% non-fat dry milk, 3% BSA, and 0.1% TBS-Tween20 at room temperature. They were then hybridized overnight at 4°C with a custom rabbit polyclonal antibody to CHFR at a working dilution of 1:1000 (N-terminal epitope: MERPEEGKQSPPPQPWGRLLRC; BioCarta, San Diego, CA) followed by a one-hour incubation at room temperature with goat anti-rabbit:HRP secondary antibody at 1:10,000 (Sigma-Aldrich, St. Louis, MO). The loading control was assessed with mouse ascites anti-β-Actin at a dilution of 1:5000 and goat anti-mouse:HRP secondary antibody at 1:20,000 (Sigma-Aldrich, St. Louis, MO) in 3.0% BSA and 0.05% TBS-Tween-20. The Super Signal West Pico Chemiluminescent kit (Pierce, Rockford, IL) was used for detection and blots were exposed to Kodak Biomax XAR film. Relative expression of CHFR was assessed by using the IS-1000 Digital Imaging System (Alpha Innotech Corp, San Leandro, CA) for densitometry to determine signal intensity, then a ratio of CHFR:β-Actin was calculated and normalized to the expression of all IHMEC cell lines. CHFR expression was considered low if the relative expression was less than 0.1, which was the lowest value among the IHMEC lines.

For Western blots to detect loss of CHFR after shRNA or siRNA, following one hour of incubation in a blocking solution of 5% non-fat dry milk and 0.1% TBS-Tween20, a monoclonal antibody against CHFR (Abnova, Taipei, Taiwan) was used at a 1:500 dilution in 5% BSA and 0.05% TBS-Tween20 and incubated overnight at 4°C. CHFR was detected by hybridization with a goat anti-mouse:HRP secondary antibody at a 1:5000 dilution in 5% BSA and 0.05% TBS-Tween20. For a loading control, blots were blocked in 5% non-fat dry milk and 0.1% TBS-Tween20 for one hour. Then, an anti-GAPDH antibody (Abcam, Cambridge, MA) was used at a 1:5000 dilution and detected with a goat anti-mouse:HRP antibody at a 1:10,000 dilution, both in 5% non-fat dry milk and 0.05% TBS-Tween20.

RT-PCR

For semi-quantitative duplex RT-PCR, reaction conditions were optimized as previously described (27). Briefly, primer concentrations were optimized to create equal band intensity between *CHFR* and the internal *GAPDH* loading control, and the cycle number that resulted in the logarithmic phase of product generation was determined. Total RNA was isolated from BCCs and IHMECS via the Qiagen RNeasy RNA isolation kit. cDNA was then generated from 1.0ug of total RNA using the Qiagen Omniscipt Reverse Transcription kit (Qiagen Inc., Valenica, CA.) and random hexamer primers. CHFR cDNA was amplified with the primers (forward/reverse, 5'-3'): CAGCAGTCCAGGATTACGTGTG/AGCAGTCAGGACGGGATGTTAC (500bp) or TCCCCAGCAATAAACTGGTC/GTATGCCACGTTGTGTTCCG (205bp) and GAPDH cDNA was amplified with the following primers (forward/reverse, 5'-3'): AGTCCATGCCATCACTGCCA/GGTGTCGCTGTTGAAGTCAG (340bp). PCR products were separated on a 1.0% agarose gel in 1x TBE and stained with ethidium bromide. Band intensity was assessed using the IS-1000 Digital Imaging System (Alpha Innotech Corp, San Leandro, CA)

For quantitative RT-PCR, cDNA samples from IHMECs and breast cancer cell lines were amplified in triplicate from the same total RNA sample following the manufacturer's instructions. Samples were amplified using TaqMan MGB FAM dye-labeled in an ABI7900HT model Real-Time PCR

machine (Applied Biosystems, Foster City, CA). To amplify CHFR cDNA, probe set Hs00217191_m1 was utilized while the control, GAPDH, was amplified with probe set Hs99999905_m1 (Applied Biosystems, Foster City, CA).

Tissue Samples and Immunohistochemistry

Since the polyclonal antibody for CHFR worked poorly for immunohistochemistry, the monoclonal anti-CHFR antibody was used at a 1:50 dilution for hybridization to paraffin-embedded sections of human breast tissue using standard methods. Primary antibody was detected following protocols described by the manufacturer (DAKOCytomation, Carpinteria, CA), using diaminobenzidine as a chromogen and with Harris Hematoxylin counterstain (Surgipath Medical Industries, Richmond, IL). Optimization and validation of the immunostaining conditions was performed on multi-organ tissue microarrays using a DAKO Autostainer.

To study CHFR expression in primary breast cancers, 160 patient samples arrayed on a single high density tissue microarray (TMA) were used for the analysis (28). Details on this TMA have been previously described (29). Tissue cores from 98 patients with invasive breast carcinoma were available to evaluate CHFR staining. The staining was scored using a 4 tiered scoring system (1=negative, 2=weak, 3=moderate, 4=strong) by pathologist, C. K. The Wilcoxon rank test was used to determine if there was an association between CHFR staining and clinico-pathological variables including patient age, tumor size, tumor grade, lymph node status, estrogen receptor (ER), progesterone receptor (PR), HER2/neu status, and patient survival.

Growth Curve Analysis

To determine the growth rate of the cellular population, $4x10^4$ cells were plated into each well in 6-well plates. Cells from three different wells were then manually counted with a hemacytometer. A new set of three wells were counted every two days for a total of 7 or 9 days, at which point at least one cell line began to reach confluence. Average cell numbers from the three wells were then plotted as a function of a time.

Immunofluorescence and Mitotic Index

Early mitotic chromosomes were identified via immunofluorescence using Histone H3-phospho-Ser28 antibody (Upstate, Lake Placid, NY) at a 1:100 dilution and anti-rabbit Alexafluor488 secondary antibody at a 1:50 dilution. Cells were blocked in 1% BSA, 0.025% TritonX-100 solution in PBS for one hour prior to incubation with primary antibody. Cells were counterstained with phalloidin conjugated to Alexafluor568 to detect the actin cytoskeleton and ProLong Gold anti-fade reagent with DAPI to detect all nuclei. Cells were visualized using a compound Leica DMRB miscroscope with a Leitz laser at 63x magnification (W. Nuhsbaum, Inc., McHenry, IL) and an Optronics camera system (Goleta, CA). The mitotic index was calculated as the number of H3-Ser28 stained nuclei from 1000 total nuclei and then converted to a percentage.

Matrigel Invasion Assay

This invasion assay was performed according to manufacturer's instructions (BD Biosciences, Bedford, MA). In short, $2.5x10^4$ cells suspended in media without chemoattractant were plated in triplicate in Matrigel baskets in a 24-well plate. In the chamber below the baskets, either media without chemoattractant as a negative control or media containing chemoattractant was added. Chemoattractants for each cell line are: (1) HPV4-12 cells: 5% FBS, 1.0 ug/ml hydrocortisone, 10.0 ug/ml insulin, 100.0 ng/ml cholera toxin, and 10.0 ng/ml epidermal growth factor, (2) MCF10A cells: 10% horse serum, 0.5 ug/ml hydrocortisone, 100.0 ng/ml cholera toxin, 10.0 ug/ml insulin, and 20.0 ng/ml epidermal growth factor, and (3) Hs578T cells: 10% FBS and 10.0 ug/ml insulin.

Cells were incubated for 22 hours at 37°C in 5% CO_2 for MCF10A and Hs578T cells or 10% CO_2 for HPV4-12 cells. The interior of the chambers were cleaned and the cells on the exterior were fixed and stained using the PROTOCOL Hema 3 staining kit (Fisher Scientific Co, Middletown VA). The number of stained cells that had traveled through the Matrigel collagen matrix was counted using a Nikon TMS inverted microscope at 10x magnification.

Scrape Motility Assay

Cells were grown to confluency in 6-well plates and the cell monolayer was mechanically scarred using a plastic pipette tip. Cells were visualized for movement into the scratched surface with a Leica DMIRB inverted microscope with phase contrast optics and a 10x objective lens. Images were captured with a SPOT camera system (Diagnostic Instruments Inc, Sterling Heights, MI). The motility phenotype was quantified by using the ImageQuant v5.2 software package (GE Healthcare/Amersham Biosciences, Piscataway, NJ) to determine the area of the initial scrape and then the area of the same wound 24-48 hours later. Images for MCF10A and Hs578t cells were captured 24 hours after the initial wound whereas HPV4-12 cells were photographed 48 hours later. Data are presented as the percentage of the scraped area that remains after the end-point.

Cellular Morphology

Cellular morphology was recorded when cultured cells reached 100% confluence. Images were gathered using a Leica DMIL inverted microscope (W. Nuhsbaum, Inc, McHenry, IL) at 10x magnification and a SPOT RT Color camera with SPOT Advanced digital imaging software (Diagnostic Instruments Inc, Sterling Heights, MI).

Ploidy Status and Nucleolar Changes

Cells were collected at 70% confluence by trypsinization and resuspended in 0.075M KCl on ice for 30 minutes. Cells were fixed in a 3:1 mixture of methanol and glacial acetic acid with mild vortexing, dropped onto glass slides, and stained with 544ug/ml Giemsa solution. To determine ploidy, the number of chromosomes was counted in at least 25 metaphases for each cell line and its derivatives.

To assess nucleolar changes, cells were prepared as described above and the number of nucleoli was counted in at least 50 cells, in triplicate, for each cell line. For both methods listed here, images were recorded with a compound Leitz DMRB miscroscope (W. Nuhsbaum, Inc., McHenry, IL) at 40x magnification and an Optronics camera (Goleta, CA).

Statistical Analysis

The ANOVA test was used to determine statistical significance when comparing quantitative phenotypic differences between parental IHMEC cells and their corresponding control shRNA/siRNA or CHFR shRNA/siRNA constructs. The Wilcoxon signed-rank test and the Student's t-test were performed to assess statistical significance when analyzing patient data from the tissue microarray. For both tests, statistical significance was defined as $p \leq 0.05$. Error bars in the graphs presented here represent the standard error of the mean (SEM).

Results

CHFR Expression in Breast Cancer Cell Lines

Few studies have analyzed the expression of CHFR protein in tumors or cell lines, partially due to the lack of a sensitive and specific commercially-available antibody; therefore, we generated a polyclonal antibody against the N-terminus of CHFR. Using this antibody for Western blot analysis, we noted variable expression with some cell lines showing low expression and some high expression. Densitometry analysis revealed that 37.5% (9 of 24) of asynchronous breast cancer cell lines appeared to have low or no CHFR expression compared to five IHMEC cell lines, whereas 20.8% (5 of 24) had expression higher than the range calculated for the IHMEC lines (Fig. 1A). The remaining lines had expression levels that fell within the range of IHMEC cells.

Previous reports indicated that *CHFR* mRNA was low in 50% of breast cancer cell lines as assessed by Northern blot analysis (30). In this study, quantitative RT-PCR was employed to better define the levels of *CHFR* mRNA from asynchronous breast cancer cell lines compared to IHMECs. RNA was collected from cells at 70-80% confluency, the same confluency used for Northern blot analysis. Quantitative RT-PCR revealed that 17% of breast cancer cells show *CHFR* expression levels significantly lower than IHMECs (Fig. 1B). The difference between Northern blot analysis and quantitative RT-PCR may be due to the much higher sensitivity of quantitative RT-PCR to low amounts of sample or perhaps some transcripts were more easily detected by the quantitative RT-PCR probe in comparison to the probe used for Northern blotting. There was no statistically significant correlation

between mRNA, as assessed by quantitative RT-PCR, and protein levels of CHFR in each cell line (R^2 = 0.1). This suggests that CHFR protein expression may be altered by post-transcriptional or post-translational modification.

CHFR Expression in Primary Breast Cancers

From 160 patient samples of invasive breast carcinoma present on the tissue microarray (TMA), 142 were available to score for CHFR staining and 98 had complete clinico-pathological data for statistical analysis. Of the 142 patient samples scored for CHFR staining, 36% were negative, but only 0.5% showed strong CHFR staining. The number of patient samples per staining score are as follows: negative (1), 51; weak (2), 35; moderate (3), 48; and strong (4), 8 (Figure 2A). Patient samples were annotated for several clinico-pathological variables including: tumor size, estrogen receptor (ER) status, progesterone receptor (PR) status, HER2/neu expression, lymph node status, patient age, and tumor grade. Interestingly, there was a trend towards CHFR staining being correlated with ER-positive tumors ($p=0.0903$ by Wilcoxon rank test, $p=0.0653$ by t-test; Figure 2B). There was a striking significant correlation between positive CHFR staining and small (< 2cm) tumor size ($p=0.0179$, Wilcoxon rank test).

Stable Loss of CHFR Results in Increased Growth Rates and Mitotic Index

CHFR expression was significantly decreased using a stably expressed shRNA construct, as determined by Western blotting and semi-quantitative duplex RT-PCR, in two IHMEC lines: HPV4-12 and MCF10A (Fig. 3A). Stable expression of shRNA reduced the amount of CHFR protein by at least 60% in HPV4-12 cells and by nearly 80% in MCF10A cells, and reduced the amount of mRNA by about 70% as determined by densitometry.

We first noticed that when CHFR expression was decreased by shRNA, the population growth rate dramatically increased for both IHMECs by at least three-fold over the course of 7-9 days ($p\leq0.03$ for MCF10A and $p\leq0.001$ for HPV4-12; Figure 3B). In order to understand this increase in population growth, we assessed the percentage of mitotic cells by using immunofluorescence to stain cells for

phosphorylated histone H3-Ser28 – a residue that is phosphorylated during metaphase and is gradually dephosphorylated in anaphase. This phosphorylation event occurs following the previously described "CHFR checkpoint," which occurs during prophase. There was a four-fold increase in the number of H3-Ser28 stained cells in the population when CHFR expression was lowered by shRNA in the HPV4-12 cell line. This indicated that more cells went through the CHFR checkpoint, entering the later stages of mitosis even in the absence of microtubule poisons (Figure 3C and Figure S1). A greater increase in H3-Ser28 phosphorylation was observed when HPV4-12 cells were transiently transfected with a pool of four siRNAs for 72 hours prior to staining to decrease CHFR protein by approximately 95% (Figures 3A and 3C). No such results were observed for MCF10A cells with transient siRNA against CHFR, but a similar trend was observed for MCF10A cells with the stable shRNA construct, though it was not statistically significant.

The Stable Loss of CHFR Leads to Enhanced Invasive Potential and Increased Motility

To determine if decreasing CHFR expression would cause phenotypic changes reminiscent of cellular transformation, IHMECs with or without CHFR shRNA were subjected to the Matrigel invasion assay and the scrape (wound) motility assay. Surprisingly, there was a dramatic increase in the ability of the cells to invade through the Matrigel collagen matrix when CHFR expression was low: a 23-fold increase for MCF10A cells and a 5-fold increase for HPV4-12 cells ($p \leq 0.001$ for both, Figure 4A). This dramatic change was also observed after transient transfection with a pool of four siRNAs, each targeting a different locus in *CHFR*, which indicated that this phenotype is directly caused by CHFR loss and is not a result of clonal selection during culture of the stable shRNA lines (Figure 4A).

To assess changes in cellular motility, a wound was created in a confluent culture of IHMEC cells with or without CHFR shRNA. Motility was described as the percentage of the area of the initial wound that remained after a recovery period. IHMEC lines are not readily motile when their growth surface has been damaged and the remnants of the initial wound are clearly visible days later. However, when CHFR expression was decreased by stable shRNA, the cells became so motile that the wound was nearly entirely

closed 24-48 hours later (Figure 4B). This was not a function of the increased population growth rates as cells with filipodia were clearly seen in the center of the wound less than 24 hours later, which is less than the amount of time for one complete cell cycle (data not shown).

Stably Decreased Levels of CHFR Cause Morphological Changes

Normally, cells contain only one or two nucleoli in a nucleus and one frequently characterized change in cancer cells are increased number or more prominent nucleoli. In fact, changes in the number of nucleoli (>3) is strongly correlated with a negative prognosis for survival in breast cancer patients (31). Interestingly, both IHMEC cell lines exhibited a marked increase in the number of nucleoli present in the nucleus, which was defined as three or more nucleoli, when CHFR expression was knocked down by shRNA. We found that 29% of MCF10A:CHFRshRNA cells (compared to 9% for controls, $p \leq 0.001$) and 23% of HPV4-12:CHFRshRNA cells had greater than three nucleoli (compared to 13% for controls $p \leq 0.08$; Figure 4C). This change in nucleolar organization and number may indicate alterations in cellular metabolism related to proliferation, genome organization, or gene expression.

The final evidence for an acquisition of tumorigenic phenotypes following knockdown of CHFR expression was noticed only in MCF10A cells. We observed that MCF10A cells with CHFR shRNA underwent a morphological change following approximately 10 passages in culture. These immortalized mammary epithelial cells became elongated and showed more variability in cell size, which is suggestive of the epithelial-to-mesenchymal transition that is often observed during tumorigenesis (Figure 4D).

Over-expression of CHFR Reverses Tumorigenic Phenotypes in Breast Cancer Cells

In the converse experiment from above, we next determined if CHFR overexpression would have any affect on a tumorigenic breast cancer cell line, Hs578T, which has no endogenous expression of CHFR protein. Hs578T cells overexpressed *CHFR* through a stably transduced retroviral construct containing the full-length cDNA (Figure 5A). Though we did not notice a significant difference in the growth rates of these cells over-expressing CHFR, we did observe a dramatic change in invasiveness and motility. When Hs578T cells had higher CHFR levels, their ability to invade through a Matrigel collagen

matrix plummeted by 25-fold ($p \leq 0.001$; Figure 5B). In addition, Hs578T cells overexpressing CHFR showed nearly a six-fold decrease in motility using the scrape assay ($p \leq 0.001$; Figure 5C).

Stable Knockdown of CHFR Expression Leads to Genomic Instability

Since genomic instability, or aneuploidy, was previously reported for mouse embryonic fibroblasts derived from the *Chfr* knockout mouse, we assessed the ploidy status of IHMECs shortly after stable CHFR shRNA expression. Strikingly, 60-70% of the cells with low CHFR were aneuploid, as opposed to less than 5% of cells in the normally diploid parental lines (Figure 6A and 6B). For aneuploid cells, the number of chromosomes present ranged from 49 to >85. Aneuploidy was also confirmed by FACS analysis as an increase in the population of cells with greater than 4N DNA content (data not shown).

Discussion

The findings presented here contribute significantly to the characterization of *CHFR* as a tumor suppressor gene. We show that CHFR protein expression was lost in many breast cancer cell lines and primary cancers. In addition, we provide evidence that decreasing CHFR mRNA and protein using shRNA/siRNA resulted in two IHMEC cell lines acquiring phenotypes associated with malignant progression. These phenotypes included increased growth rates and mitotic indexes, the cells acquired the abilities of invasion and motility, and a striking percentage of cells became aneuploid.

There were a substantial number of cell lines and primary patient samples with very low CHFR protein expression, the percentages of which were nearly identical (37.5% vs. 36%). Intriguingly, the amount of CHFR mRNA and protein varied greatly among breast cancer cell lines and even a few appeared to overexpress CHFR. However, the number of cell lines and primary samples with low CHFR protein expression outnumbered those that exhibited high expression. In addition, the decreased protein expression of CHFR by shRNA/siRNA revealed highly significant results supporting the hypothesis that CHFR is a tumor suppressor. When CHFR was overexpressed in Hs578T breast cancer cells, the data suggested that higher CHFR levels did not have any adverse consequences and, in fact, reversed some tumorigenic phenotypes thereby further supporting the role of CHFR as a tumor suppressor. When the

CHFR expression data is combined with the results of the phenotypic analysis *in vitro* and the correlation with tumor size *in vivo*, it appears that the loss of CHFR, not its overexpression, is relevant to tumorigenesis in mammary epithelial cells.

It is particularly interesting that there is poor correlation between mRNA and protein levels for CHFR. This is highly suggestive of post-transcriptional and/or post-translational modification to regulate expression levels and activity, which is a novel finding for CHFR. Until now, previously reported studies only indicated that *CHFR* promoter hypermethylation influenced expression, an event that is not commonly observed in breast cancers (30). These potential modifications are an intriguing new avenue to pursue the study of CHFR regulation and function at the molecular level that may be relevant to cancer progression, which is highly promising since there has been a lack of mutations reported for *CHFR* and mRNA expression alone may not indicate proper CHFR function.

In regards to primary invasive breast carcinoma, the correlation between CHFR staining and small tumor size, a very important prognostic indicator, is remarkable and supports a role for CHFR as a tumor suppressor. This is consistent with observations *in vitro* in which decreased CHFR expression led to a dramatic increase in population growth rates and a higher percentage of mitotic cells. In addition, the putative association of CHFR and ER expression may provide continued support of a role for CHFR as a biomarker for breast cancer treatment. This is particularly relevant given previous clinical trials that showed ER-positive, and therefore possibly CHFR-positive, breast cancers did not respond as well to paclitaxel treatment as ER-negative breast cancers (32-34). This corresponds well with previously published work describing CHFR-negative cells as sensitive to microtubule poisons in culture, undergoing apoptosis sooner than their CHFR-positive counterparts. The weak association of expression between ER and CHFR may help to elucidate another molecular pathway in which CHFR functions to mediate cell proliferation or a common means of gene expression regulation.

Importantly, decreased CHFR expression led to an increase in the number of mitotic (metaphase and anaphase) cells in the population. Previously, this phenotype had only been described to occur in the

presence of nocodazole and was thought to be due to an impaired checkpoint. However, the fact that this phenomenon also occurs without microtubule poisons suggests that CHFR can possibly play a wider role in regulating the timing of mitotic entry. This may help explain why the growth rates were faster in cells stably expressing CHFR shRNA and why tumors from breast cancer patients are larger when CHFR staining is absent.

Some of the most striking changes that resulted from altering CHFR expression were changes in invasion and motility of cells *in vitro*. This is the first time that CHFR has been implicated in a functional role other than cell-cycle regulation. Considering its proposed role of monitoring microtubule dynamics, it is hypothesized that CHFR has an even larger part in cytoskeletal organization in which loss would more easily allow for the necessary reorganization of the cytoskeletal network required for motility. In addition, if the phenotypes observed in culture are found to mirror those seen in cancer patients (ie: patients with low CHFR tumors have a higher incidence of distant metastases), then CHFR expression may be an indicator for tumor stage and/or patient prognosis.

Our report that low CHFR expression leads to genomic instability corroborates previously published work in the mouse. These data are suggestive of a problem with the structure or function of the mitotic spindle that is not corrected due to an impaired CHFR checkpoint. However it could also indicate a defect in cytokinesis, which is plausible since work with the two yeast orthologs of CHFR show an interaction with the septin cytoskeletal network and they function in both the spindle checkpoint and cytokinesis (35, 36). Given the relatively frequent occurrence of low/lost CHFR in many types of tumors, this work may begin to explain the conundrum of the prevalence of aneuploidy in cancers but the lack of defective spindle checkpoint mediators such as the MAD and BUB proteins.

It is not surprising that the same phenotypes were not always observed in the two cell lines tested. This is likely due to the unique genetic defects that caused the immortalization of the cell lines, thereby providing a clue to the genetic and physical interactions that CHFR has within the cell. Specifically, the HPV4-12 cell line was immortalized with the HPV E6/E7 protein to inhibit p53 and pRb function while

the MCF10A line was spontaneously immortalized following a t(3;9)(p14;p21) translocation that disrupted the p15/p16 gene in addition to other chromosomal rearrangements (37, 38). The differences in alterations may help to explain why MCF10A cells undergo a morphological change after CHFR shRNA whereas HPV4-12 cells do not, and why HPV4-12 cells more readily phosphorylate histone H3-Ser28. Differences may also be attributed to the fact that these two IHMEC lines are grown in different media with different levels of CO_2, but it should be noted that the media are very similar and contain nearly identical supplements.

This work on the phenotypic changes that arise *in vitro* with CHFR expression variation provides a unique insight as to what may happen in cancer patients and presents many new avenues through which to study CHFR expression, function, and molecular interactions. We report for the first time a correlation between CHFR levels and clinico-pathological variables in primary breast cancer: tumor size and perhaps estrogen receptor status. As evidence builds, CHFR is gaining more time in the spotlight as a novel tumor suppressor as it aspires to be the next biomarker in cancer characterization.

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Figure Legends

Figure 1: CHFR protein and mRNA expression is low in a subset of breast cancer cells when compared to "normal" IHMECs. (A) Western blot analysis using a polyclonal antibody to the N-terminus of CHFR reveals that 37.5% of asynchronous cell lines (underlined) at 70-80% confluence have low CHFR expression compared to the average expression in immortalized human mammary epithelial cells (IHMECs, in bold text). To control for loading, an antibody against beta-actin was used (bottom). (B) Quantitative RT-PCR showed that 17% of breast cancer cell lines have low *CHFR* mRNA levels compared to mRNA levels in IHMECs.

Figure 2: CHFR levels are low or negative in many primary breast cancers and correlates with tumor size. (A) Immunohistochemistry using a monoclonal antibody against CHFR on primary invasive breast cancers from a tissue microarray shows a range of CHFR expression. Intensity of CHFR staining ranged from negative (1) to weak (2), moderate (3), and strong (4). The images in the top row are at 10x magnification whereas the corresponding pictures in the bottom row are 40x magnification from sections of the images above. (B) Statistical analyses of clinico-pathological characteristics from 142 primary invasive breast carcinoma samples indicate that positive CHFR expression correlates strongly with small (<2 cm) tumor size and has a weaker association with estrogen receptor positive (ER+) status. P values were calculated using the Wilcoxon rank test, except for tumor grade (*) for which the p value was calculated using Student's t-test.

Figure 3: Decreasing CHFR expression using shRNA and siRNA in IHMECs leads to increased population growth rates and a higher number of cells entering metaphase. (A) Top: Western blotting shows a dramatic loss of CHFR protein following stable shRNA expression by retroviral transduction and transient siRNA transfection. HPV4-12 with CHFR shRNA3.1 had at least a 60% decrease while MCF10A with CHFRshRNA123 showed nearly an 80% stable knockdown of CHFR expression. Transient siRNA transfection resulted in a 95% decrease in HPV4-12 cells and an approximately 99% decrease in MCF10A cells. Bottom: Semi-quantitative duplex RT-PCR indicates a corresponding decrease in mRNA levels for each cell line. (B) Growth curves for HPV4-12 cells (left) and MCF10A cells (right) following stable shRNA expression (▲) compared to the parental cell lines (♦) and the scramble shRNA (■) negative control cell lines. Cells were counted in triplicate every 2 days until at least one line reached confluency. The graph represents the average number of cells counted on each day per cell line. One asterisk (*) indicates $p \leq 0.001$ and two (**) indicates $p \leq 0.03$, as determined by ANOVA (C) The mitotic index of cells with or without CHFR shRNA is represented as the percentage of Histone H3-Ser28 stained nuclei, indicating early metaphase through anaphase cells, from 1000 total (DAPI-stained) nuclei from each cell line.

Figure 4: Decreasing CHFR expression using shRNA and siRNA in IHMECs leads to dramatic increases in invasive potential and motility, in addition to morphological changes. (A) Both stable (top) and transient (bottom) knockdown of CHFR expression results in greatly increased invasive potential through a Matrigel collagen matrix for both HPV4-12 cells (left) and MCF10A cells (right). Asterisk (*) indicates $p \leq 0.001$. Top left: HPV4-12 with stable CHFR shRNA. Top right: MCF10A with stable CHFR shRNA. Bottom left: HPV4-12 with transient CHFR siRNA. Bottom right: MCF10A with transient CHFR siRNA (B) Left: Digital phase contrast images at 10x magnification showing an increase in motility (closing a scraped wound in confluent culture) for HPV-12 (far left) and MCF10A (middle) cells following stable CHFR shRNA expression. The “before” images depict the initial wound in the culture and the “after”

images show wound closure after 24 hours (MCF10A) or 48 hours (HPV4-12). Right: Graphical representation of the degree of wound closure depicted on the left. Motility is described as the percentage of the original wounded area that remains vacant after incubation. Asterisk (*) indicates $p \leq 0.001$, calculated using ANOVA. (C) Left: Giemsa-stained cells in which the nucleolus is depicted as a dark spot within the nucleus. Parental (far left) and scramble shRNA controls (middle) normally contain one or two nucleoli whereas CHFR shRNA cells more frequently had greater than three nucleoli (arrows). Right: Quantification of the percentage of cells with greater than three nucleoli, n=50 per trial, for each cell line. Asterisk (*) means $p \leq 0.001$ (ANOVA). (D) MCF10A cells visualized by light microscopy showing change in cellular shape from epithelial to an elongated morphology reminiscent of an epithelial to mesenchymal transition.

Figure 5: Stably increasing CHFR by retroviral transduction of a full-length CHFR cDNA construct in a breast cancer cell line, Hs578T, rescues some malignant phenotypes. (A) Western blot showing increased CHFR expression (top) in cells retrovirally transduced with a Flag-tagged CHFR construct. Beta-actin is used as a loading control (bottom). (B) Over-expression of CHFR results in the loss of invasive potential through a Matrigel collagen matrix. Asterisk (*) indicates $p \leq 0.001$. (C) Left: Phase contrast images at 10x magnification showing an increase in motility for Hs578T cells following stable CHFR over-expression. The “before” images depict the initial wound in the culture and the “after” images show wound closure after 24 hours. Right: Graphical representation of the degree of wound closure depicted on the left; the graph describes the percentage of the original scraped area remaining after incubation for each cell line. Asterisk (*) indicates $p \leq 0.001$ calculated by ANOVA.

Figure 6: Decreased CHFR expression causes genomic instability. (A) Giemsa-stained metaphase spreads of parental, negative control, and CHFR shRNA cells. IHMECs with lowered CHFR expression showed a greatly increased incidence of aneuploidy (>48 chromosomes). (B) Quantification of aneuploidy in

CHFR shRNA cells showing that low CHFR expression results in 55-72% of the cells in the population becoming aneuploid. The graph represents the percent of aneuploid cells, from 25 counted metaphases, for each cell line.

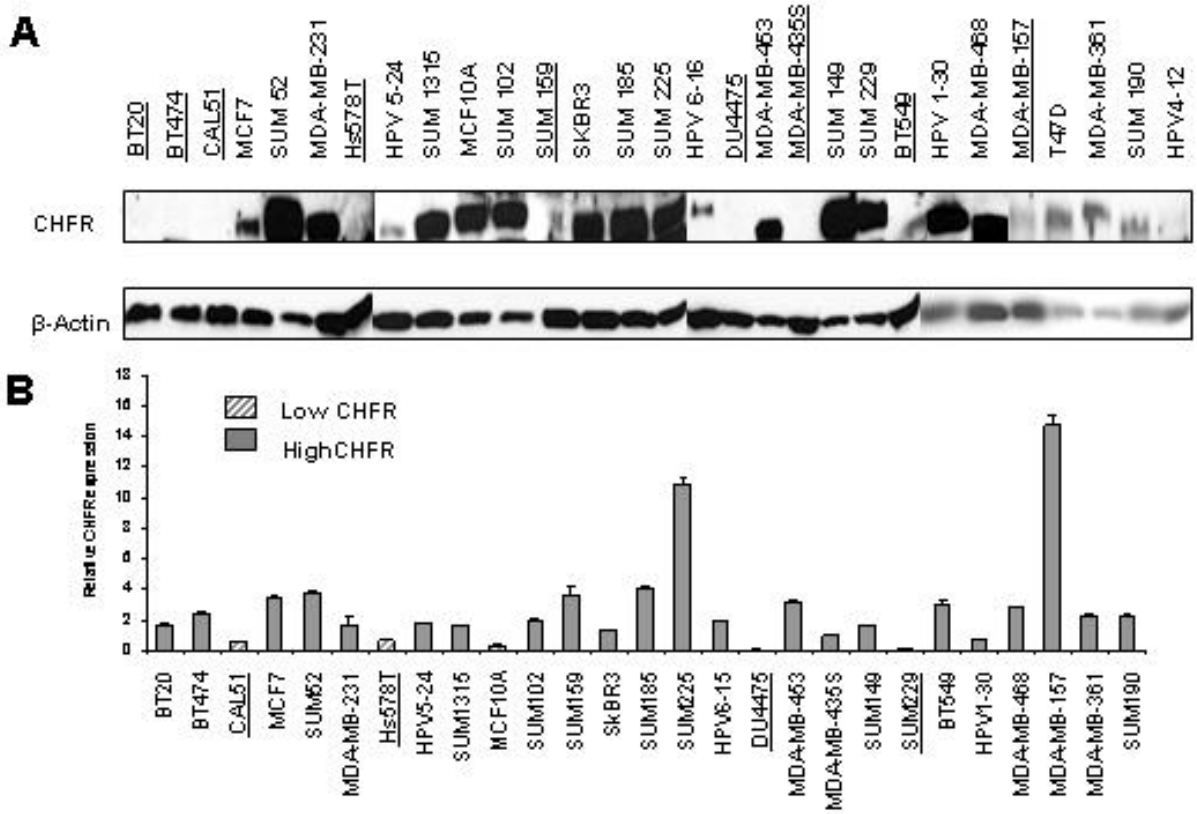
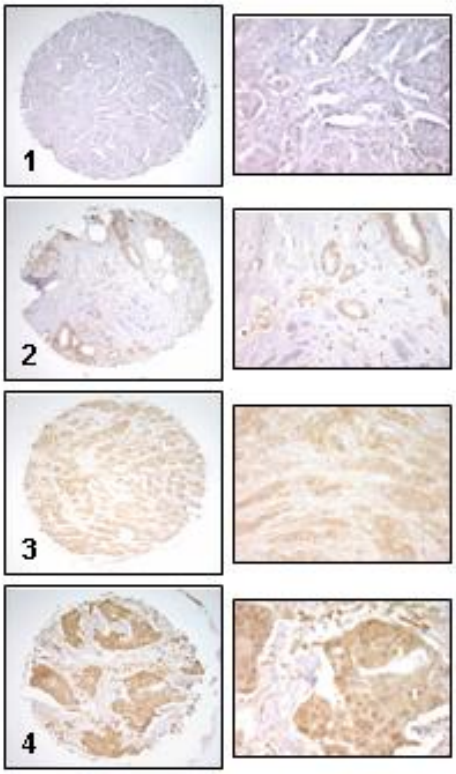


Figure 1, Privette

A



B

Correlation of CHFR expression with clinico-pathological variables from 160 patient samples

Clinico-pathological variables	Number of patients with variable	P value*
Age		
<50	47	0.9350
>=50	96	
Size		
< 2 cm	80	0.0179
≥ 2 cm	63	
Positive Lymph Nodes		
High (>6)	24	0.3814
Low (1-3)	111	
ER expression		
Positive	87	0.0903
Negative	44	
PR expression		
Positive	71	0.8381
Negative	61	
Her2/neu		
Positive	122	0.9353
Negative	21	
Grade		
1	13	0.8043**
2	63	
3	65	

* Wilcoxon rank test for significance ** Student's t-test0

Figure 2, Privette

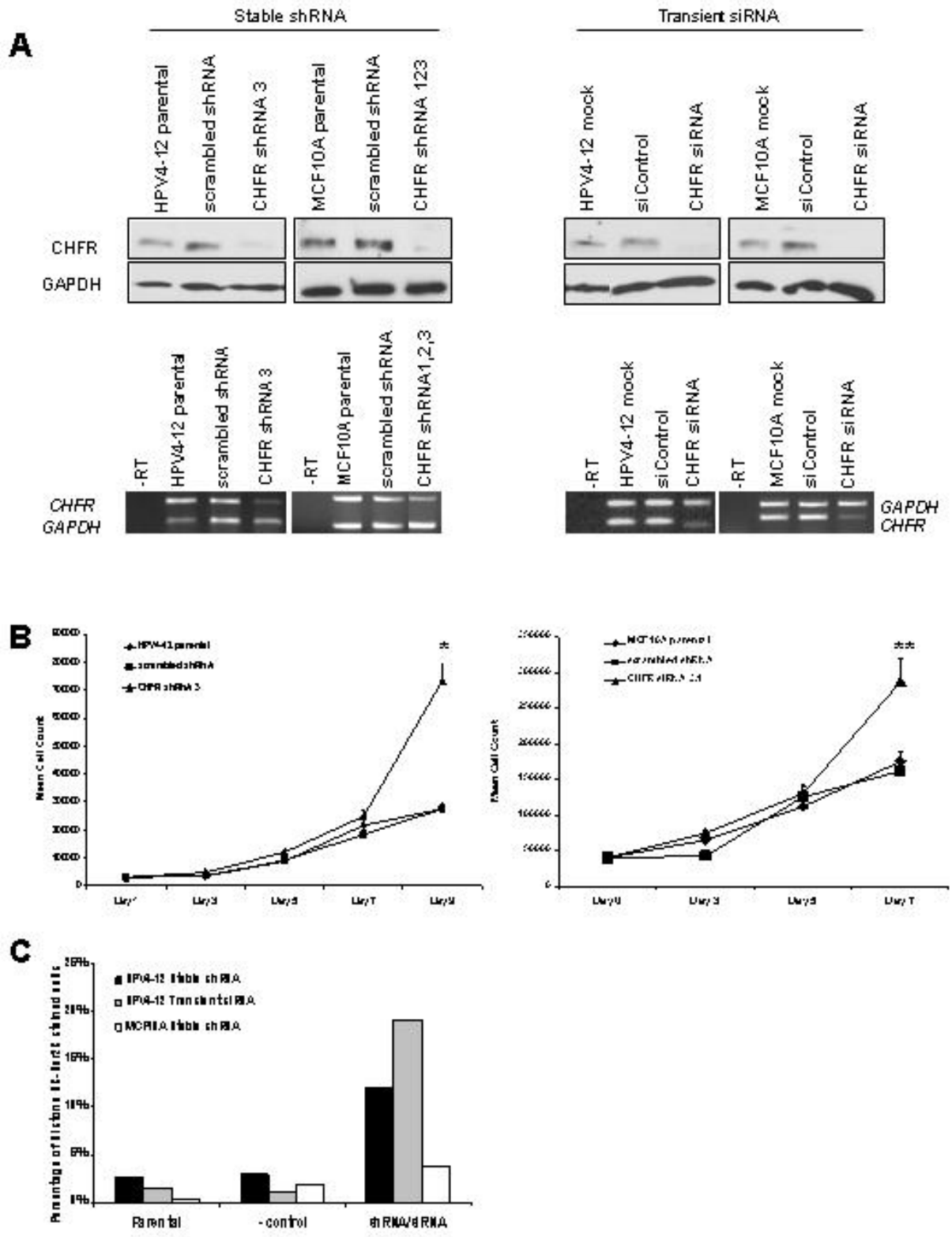


Figure 3, Privette

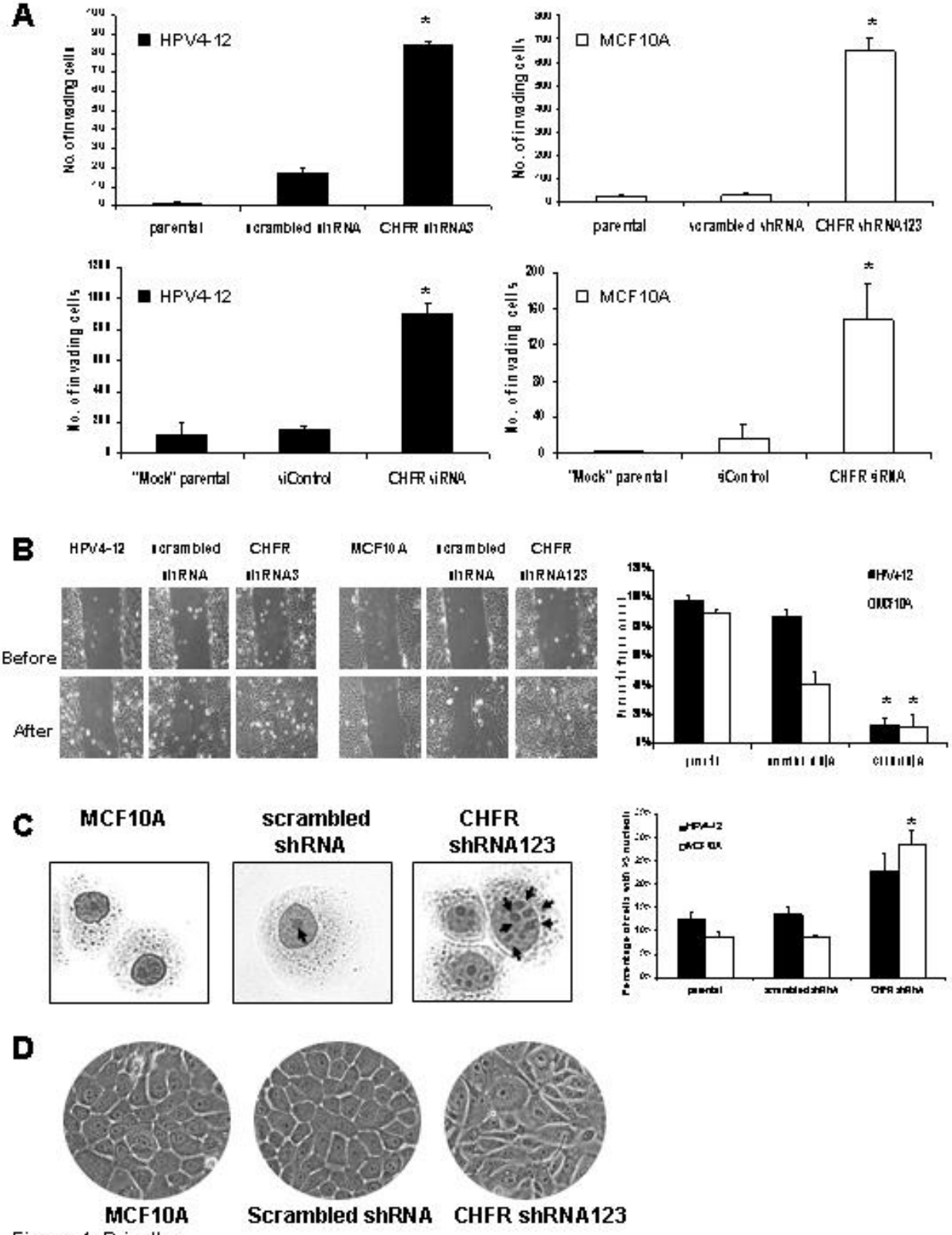


Figure 4, Privette

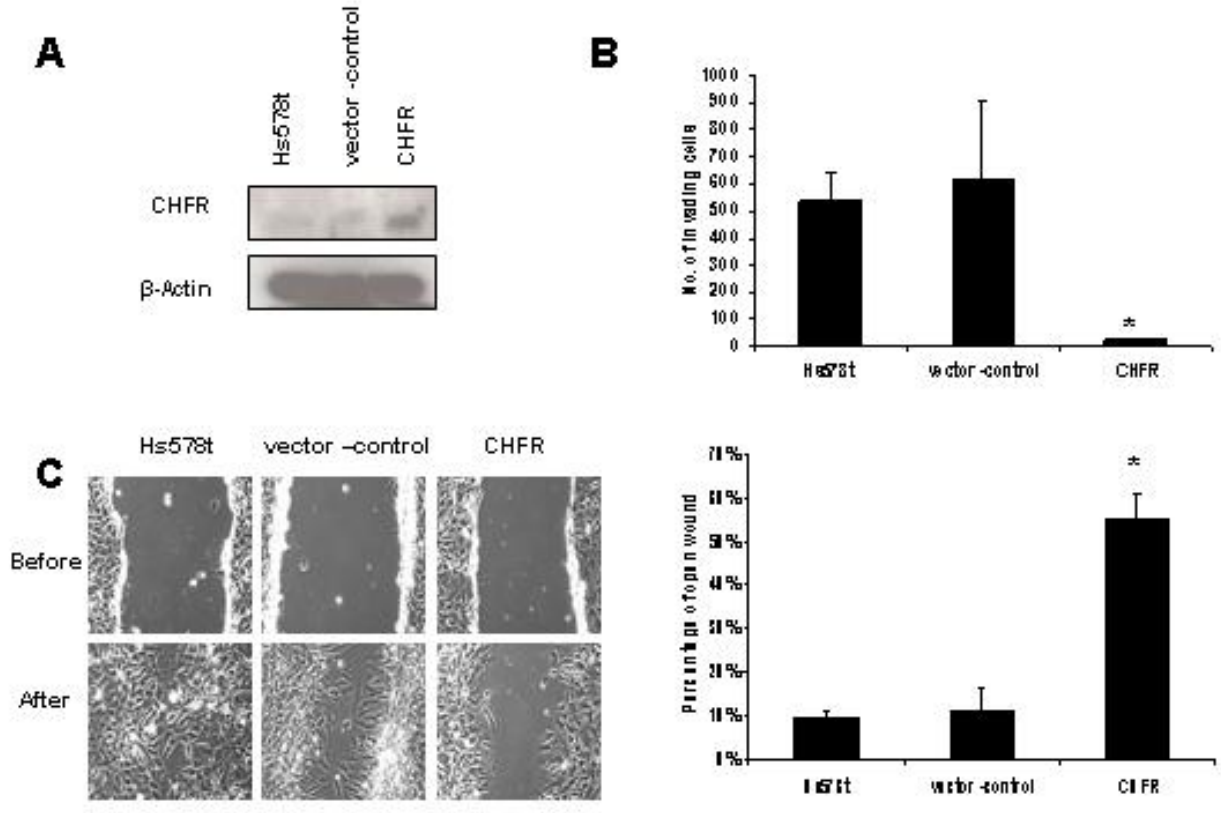


Figure 5, Privette

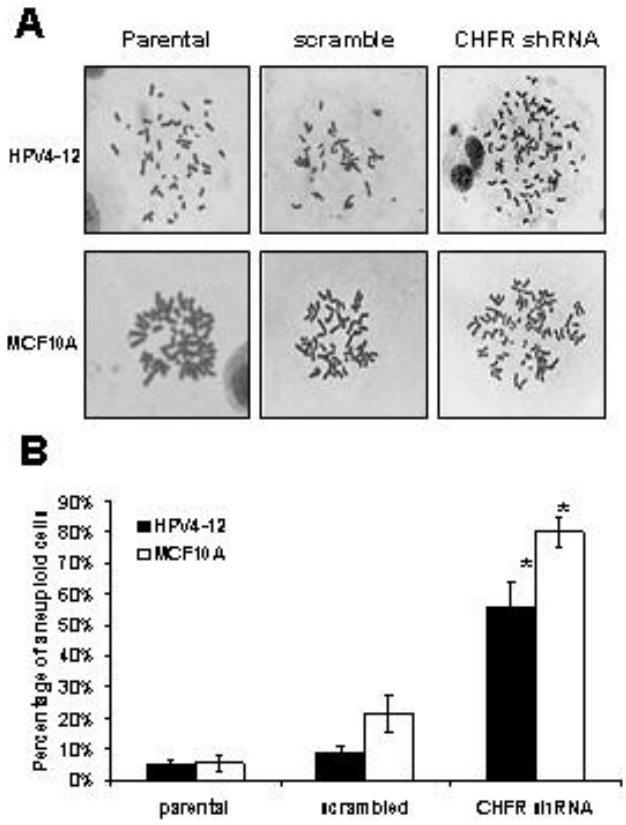


Figure 6, Privette